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Note

Bile acid ethyl esters

Their infrequent formation during routine bile acid analysis and identification by gas chromatography and mass spectrometry*

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During the gas–liquid chromatographic (GLC) analysis of the guinea-pig fecal bile acids by a procedure described previously from this laboratory[1] using hyocholic acid as an internal standard, we noticed infrequently the appearance of two unknown peaks (one with a relative retention time of 1.19, relative to lithocholic acid methyl ester trifluoroacetate, and the other with a relative retention time of 1.17, relative to hyocholic acid methyl ester trifluoroacetate) in the chromatogram. These peaks did not correspond to any known bile acid that could be formed by bacterial action from chenodeoxycholic acid, which is the predominant biliary bile acid in the guinea-pig[2]. Hence, efforts were made to identify the peaks by combined gas–liquid chromatography and mass spectrometry (GLC–MS). This resulted in the identification of these two peaks as the ethyl esters** of lithocholic and hyocholic acids, respectively. Further studies also showed that these were the minor products formed infrequently during the analytical step in which the bile acids are heated with ethanolic NaOH. This is done routinely for bile acid deconjugation[3,4].

METHODS

Guinea-pig fecal pellets were homogenized with six volumes of water in a paint mixer. To about 3 mg of fecal homogenate was added 1 ml of 1 mg/ml hyocholic acid in ethanol. The mixture was then subjected to mild saponification (20 ml of 1 *N* NaOH in 90% ethanol) at 110° for 1 h using a Temp-Blok module heater (Lab-Line Instruments, Melrose Park, Ill., U.S.A.). The neutral sterols were extracted with petroleum ether, (b.p. 35–60°) as described by Miettinen *et al.*[5]. Two milliliters of 10 *N* NaOH and 5 ml of ethanol were added to the aqueous phase, and the mixture was boiled at 110° for 3 h. After cooling, the mixture was brought to pH 3 with concentrated HCl. After additional cooling, the mixture was extracted with chloroform–methanol (2:1) and

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chloroform[1,6]. The combined extracts were evaporated to dryness and the residue was dissolved in 20 ml of methanol. Aliquots of the samples were methylated with diazomethane[1,6]. The bile acid methyl esters were then converted into trifluoroacetates, as described previously[1,6,7]. GLC of the bile acid methyl ester trifluoroacetates was carried out using an F & M Model 402 high-efficiency gas chromatograph, equipped with a glass column (4 ft. × 3 mm I.D.) packed with 3% QF-1 on 100–120 mesh Chromosorb W; the operating conditions were: column temperature 215°; flash heater temperature 240°; detector temperature 250°; carrier gas, helium; flow-rate, 50 ml/min.

Combined gas–liquid chromatography and mass spectrometry

Mass spectrometry (MS) was carried out on a Varian MAT CH-5 single focusing mass spectrometer coupled with a Varian Data 620i computer[8]. The GLC separations were obtained on a Varian Model 2700 Moduline gas chromatograph equipped with a 3 ft. × 2 mm I.D. glass column packed with pretested QF-1 (3)% on 80–100 mesh Gas-Chrom Q. The carrier gas was helium. The gas chromatograph, which did not have a separate detector, was run at 225° isothermally, with the injector at 210°. The transfer line and the Watson–Biemann dual-stage helium separator were kept at 275°.

RESULTS

Fig. 1 shows the GLC elution patterns of the guinea-pig fecal bile acids as methyl ester trifluoroacetates on QF-1 columns. The presence of the unknown peaks

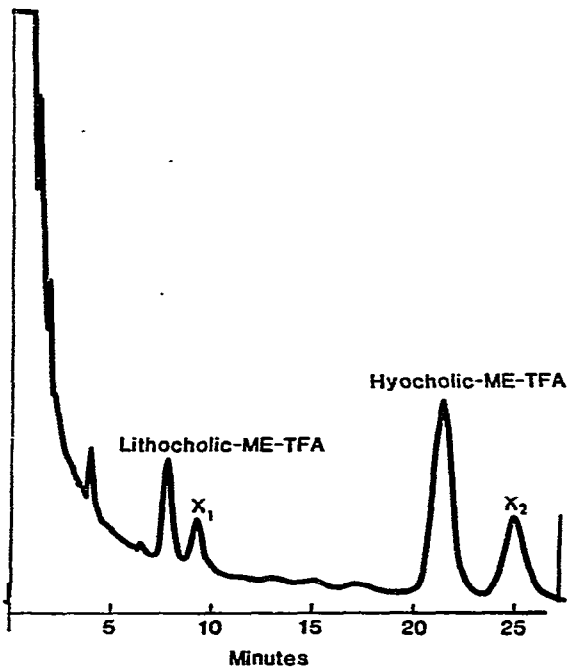

Fig. 1. Gas–liquid chromatogram of guinea-pig fecal bile acids as methyl ester trifluoroacetates on 3% QF-1 column. X_1 and X_2 are unknown peaks. Column conditions, as described in text.

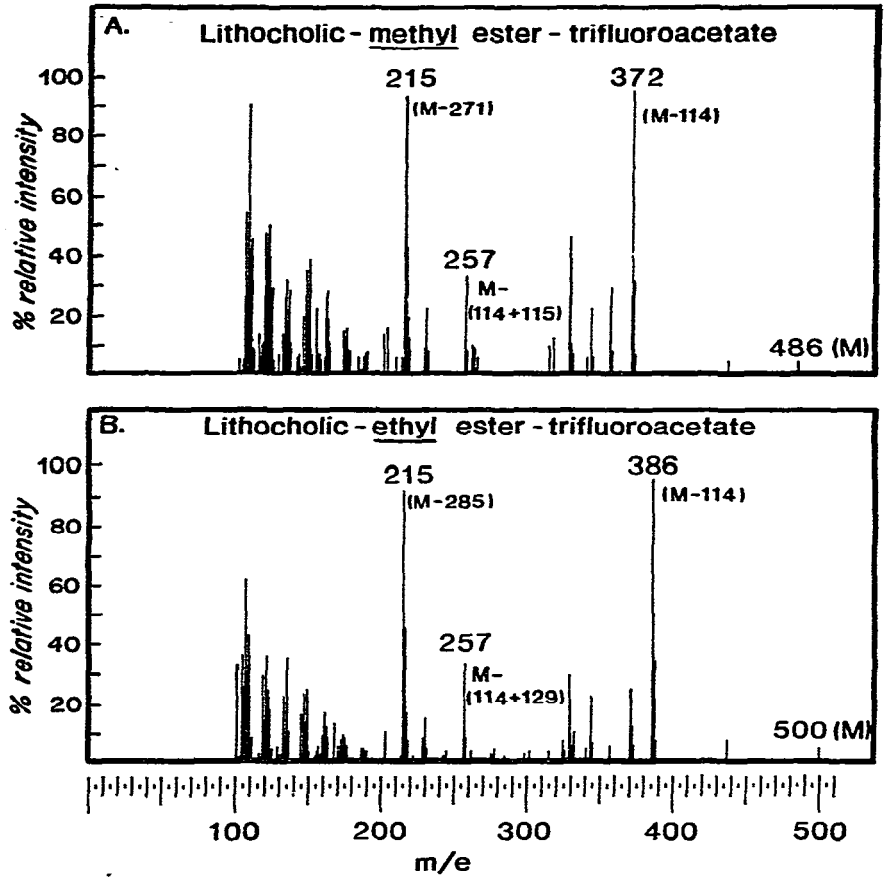

Fig. 2. Mass spectra of trifluoroacetate of lithocholic acid methyl ester (A) and unknown peak X_1 (B). Conditions for GLC–MS, as described in text.

X_1 and X_2 can be seen in the figure; the relative retention times were 1.19 and 1.17, relative to lithocholic and hyocholic acid methyl ester trifluoroacetates, respectively. These peaks were then examined by MS (Fig. 2). When the fragmentation pattern of these peaks was compared with that of lithocholic and of hyocholic acid methyl ester trifluoroacetates, it became evident that these peaks contain an ethyl (C_2H_5) group rather than the normal methyl (CH_3) group that is introduced during the methylation step in bile acid analysis[1]. The peak corresponding to the retention time of lithocholic acid methyl ester trifluoroacetate gave a molecular ion at *m/e* 486, characteristic of the monohydroxycholanates. It had a major peak at *m/e* 372 (due to the loss of trifluoroacetate), at *m/e* 329 (due to the loss of side chain + C_{15}, C_{16}, C_{17} + H), and at *m/e* 257 (due to the loss of trifluoroacetate and side chain), and its mass spectrum corresponded well with that of authentic lithocholic methyl ester trifluoroacetate. The peak X_1 (with a retention time of 1.19 min, corresponding to lithocholate methyl ester trifluoroacetate), however, gave a molecular ion at *m/e* 500, which is 14 mass units higher than 486 (lithocholate). This reinforced the possibility of the presence of an ethyl group (14 mass units higher) rather than the usual methyl group. The major

fragments derived after cleavage of the side chain were similar to the methyl ester trifluoroacetate of lithocholic acid (as was to be expected), but the fragments derived due to the loss of trifluoroacetate were 14 mass units higher than the fragments obtained for lithocholic acid methyl ester trifluoroacetate (*m/e* 386 instead of *m/e* 372). This indicates that the unknown peak is the ethyl ester of lithocholic acid.

Similarly, the peak X_2 (with a relative retention time of 1.17 min, corresponding to hyocholate methyl ester trifluoroacetate) gave a molecular ion at *m/e* 724, 14 mass units higher than that of hyocholic acid methyl ester trifluoroacetate (*m/e* 710). Whereas the hyocholic acid methyl ester showed an M−114 (less of a trifluoroacetate) at *m/e* 596, the ethyl ester showed a peak at *m/e* 610. The fragments, which included ions from the loss of side chain, were similar (*m/e* 367) in hyocholic acid methyl ester and hyocholic acid ethyl ester.

The formation of ethyl esters was further documentedwhen the original samples that contained the extra peaks were saponified with aqueous NaOH, extracted, and subjected to GLC after diazomethylation and trifluoroacetation. As seen in Fig. 3, the peaks disappeared completely.

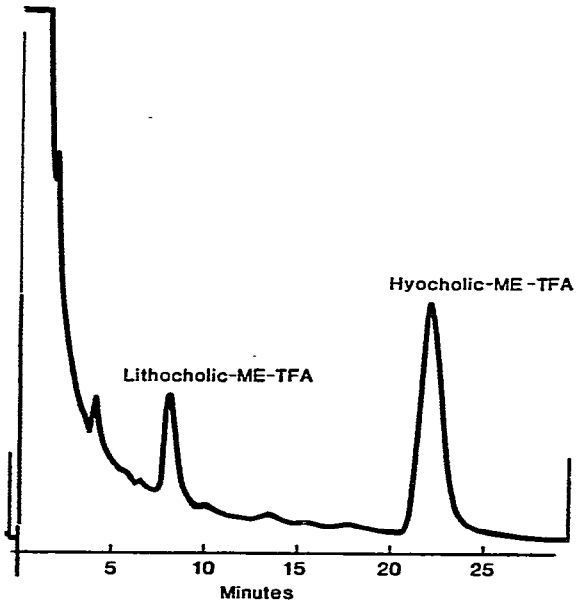

Fig. 3. Gas–liquid chromatogram of guinea-pig fecal bile acids as methyl ester trifluoroacetates after re-saponification of original sample to hydrolyze bile acid ethyl esters formed. Column conditions, as described in text.

One of the steps that seemed critical in minimizing the formation of the ethyl ester was the acidification after saponification. We found that the formation of the ethyl ester was more likely when the mixture was acidified to pH <1 rather than to pH 3, which is normally recommended. We also found that the addition of water to the hot saponification mixture seems to hydrolyze the ethyl esters formed and hence eliminates their occurrence on GLC.

DISCUSSION

This study has demonstrated the formation of ethyl esters of bile acids during the saponification with ethanolic NaOH used routinely to hydrolyze the bile acid conjugates. The formed ethyl esters were identified by GLC–MS. This study has also shown that it is necessary to identify all the peaks properly because it is possible to mistake these ethyl ester peaks for other bile acids. This is especially important if the internal standard used also gives rise to similar esters. This can result in serious miscalculations of the data. We recommend that the pH of the saponification mixture after acidification should not be allowed to drop below 3 because such a decrease seems to enhance the formation of ethyl esters. Moreover, adding water to the warm saponification mixture seems to hydrolyze the ethyl esters of bile acids (if any have formed) and thus eliminates this problem. We strongly recommend that the bile acid peaks that appear on GLC be identified carefully, especially when chromatographing has complicated the bile acid mixtures. Further studies on the conditions that favor the formation of ethyl esters of bile acids during bile acid analysis are needed.

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REFERENCES

1 M. T. R. Subbiah, *J. Lipid Res.*, 14 (1973) 692.
2 L. J. Schoenfield and J. Sjövall, *Acta Chem. Scand.*, 20 (1966) 1297.
3 S. M. Grundy, E. H. Ahrens, Jr. and T. A. Miettinen, *J. Lipid Res.*, 6 (1965) 397.
4 B. I. Cohen, R. F. Raicht, G. Salen and E. H. Mosbach, *Anal. Biochem.*, 64 (1975) 567.
5 T. A. Miettinen, E. H. Ahrens, Jr. and S. M. Grundy, *J. Lipid Res.*, 6 (1965) 411.
6 M. T. R. Subbiah, N. E. Tyler, M. D. Buscaglia and L. Marai, *J. Lipid Res.*, 17 (1976) 78.
7 M. T. R. Subbiah, *Clin. Chim. Acta*, 48 (1973) 19.
8 J. J. Myher, L. Marai and A. Kuksis, *Anal. Biochem.*, 62 (1974) 188.